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14. ABSTRACT Human breast cancer cells with restored BRMS1 expression exhibit few in vitro changes when compared to control cells, but demonstrate a very strong suppression of metastasis in in vivo animal models of breast cancer and several other solid tumor types. We have previously shown that in tissue samples collected from breast cancer patients, there exists an inverse correlation between expression of BRMS1 and HER2, an important druggable target in breast cancer. HER2 expression and function are particularly important in the context of inflammatory breast cancer, where up to 60% of all tumors are HER2+, but usually negative for hormone receptors ER and PR. Patients with inflammatory breast cancer have few treatment options and have one of the highest metastatic relapse rate and lowest survival among all breast cancer patients. In Year 1 progress report, we identified KPL4 inflammatory breast cancer cell line as a good candidate for re-expression of BRMS1, since there is HER2 amplification and cells were described in the literature as spontaneously metastatic. In the studies described below, we show that BRMS1 expression in these cells inhibits cell adhesion to several matrices, and preferentially suppresses metastasis to bone. We also begin to investigate molecular mechanisms responsible for inhibition of metastasis and identify Stat3 signaling as a potential driver signaling cascade.					
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1. Introduction

Human breast cancer cells with restored BRMS1 expression exhibit few *in vitro* changes when compared to control cells, but demonstrate a very strong suppression of metastasis in *in vivo* animal models of breast cancer and several other solid tumor types. We have previously shown that in tissue samples collected from breast cancer patients, there exists an inverse correlation between expression of BRMS1 and HER2 (1), an important druggable target in breast cancer. HER2 expression and function are particularly important in the context of inflammatory breast cancer, where up to 60% of all tumors are HER2+, but usually negative for hormone receptors ER and PR (2). Patients with inflammatory breast cancer have few treatment options and have one of the highest metastatic relapse rate and lowest survival among all breast cancer patients (2). In Year 1 progress report, we identified KPL4 inflammatory breast cancer cell line as a good candidate for re-expression of BRMS1, since there is HER2 amplification and cells were described in the literature as spontaneously metastatic (3). In the studies described below, we show that BRMS1 expression in these cells inhibits cell adhesion to several matrices, and preferentially suppresses metastasis to bone. We also begin to investigate molecular mechanisms responsible for inhibition of metastasis and identify Stat3 signaling as a potential driver signaling cascade.

As was described in the modified Statement of Work, tasks 1.3 and 1.4 were completed previously and will not be discussed further in this progress report. Tasks 1.1 and 1.2 were initiated during Year 1, but were not completed. Therefore, we will describe our progress during Year 2 on tasks 1.1, 1.2, 2.2, 2.3, 2.4, and 2.5.

2. Results

Task 1.1.

The objective of Task 1.1 was to identify several HER2-amplified metastatic breast cancer cell lines and, if endogenous BRMS1 expression was low, to generate cell line variants expressing exogenous BRMS1. In last year's progress report we described that KPL4, an inflammatory breast cancer cell line, had been described as spontaneously metastatic to lymph nodes and lungs in both nude and SCID mice and as exhibiting a 15-fold amplification of HER2 (3). During Year 2 of the project, we have now fully characterized *in vitro* and *in vivo* behavior of KPL4 cells expressing either vector only plasmid or plasmid carrying BRMS1. As shown in Figure 1, BRMS1 expression was confirmed both on mRNA (Fig. 1a) and protein level (Fig. 1b). Since several of our proposed sub-Aims for this project require protein complex purification and BRMS1 antibody does not work well for immunoprecipitation, we constructed BRMS1 to contain a FLAG tag (Fig. 1b). Once we confirmed BRMS1 expression, we examined *in vitro* proliferation rate of vector and BRMS1-expressing cells and determined that these cell lines proliferate at a similar rate (Fig. 1c). Finally, since our overriding hypothesis for this project is that BRMS1 suppresses breast cancer metastasis by inhibiting adhesion of breast cancer cells to extracellular matrix (ECM), we tested ability of vector and BRMS1-expressing KPL4 cells to adhere to different matrices. As shown in Figure 1d, BRMS1 expression slightly inhibits adhesion of breast cancer cells to fibronectin (FN), laminin (LN), tenascin (TN), and vitronectin (VN), but not to collagens I, II, or IV. We also performed short-term adhesion studies as described in Year 1 progress report, but did not observe a similar adhesion inhibition phenotype with KPL4 cells, as compared to MDA-MB-231 and MDA-MB-435 cell lines. This is possibly due to the fact that both MDA cell lines are mesenchymal in their appearance and molecular profile, while KPL4 cells are more epithelial. On this same note, KPL4 cells were completely non-invasive in both Boyden chamber assays using either collagen I or matrigel, or in 2D wound-healing assay (data not shown).

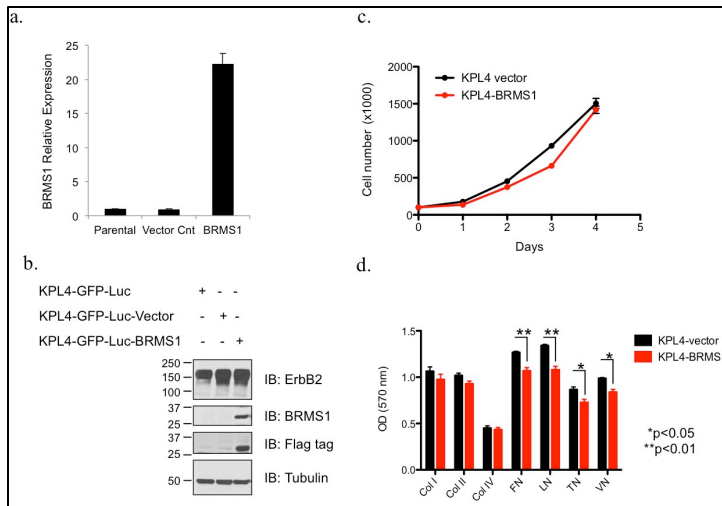


Figure 1. BRMS1 is stably expressed in KPL4 breast cancer cells. BRMS1 expression was confirmed at mRNA (a) and protein level (b). Cell proliferation (c) was assessed by cell counting and was performed in triplicate. Data are mean \pm SEM. Cell adhesion assay (d) was performed in triplicate, using a Cell Adhesion Kit from Millipore by following manufacturer's instructions. Data are mean \pm SEM. Data were analyzed by Student's t-Test.

Following *in vitro* characterization, we analyzed *in vivo* behavior of these cells. As expected, expression of BRMS1 did not affect growth of primary tumors (Fig. 2a). Primary tumor growth rate was very slow, and we did not observe development of spontaneous metastases. However, we observed unexpected results in a metastatic assay when vector or BRMS1-expressing KPL4 cells were injected intravenously. As shown in Figure 2b, there was an overall inhibition of metastases, as assessed by total bioluminescence taken throughout the experiment. Interestingly, degree of metastases suppression was not as great as it would have been expected based on prior publications. Moreover, when we counted the total number of metastatic lesions per mouse for each group, we observed no significant difference (Fig. 2c; vector: 5.2 ± 0.86 vs. BRMS1: 4.2 ± 0.66 , $p=0.19$). However, there was a very strong specific inhibition of bone metastatic lesions detected per mouse in BRMS1 group (Fig. 2d; vector: 3.4 ± 1.12 vs. BRMS1: 0.4 ± 0.24 , $p=0.01$), which was confirmed by X-ray (Fig. 2e) and histology (data not shown). These results are very encouraging because inflammatory breast cancer is one of the deadliest subtypes of the disease, with high rate of relapse and few therapeutic options available. Importantly, compared to all other subtypes of breast cancer, inflammatory breast cancer has the highest rate of bone metastases.

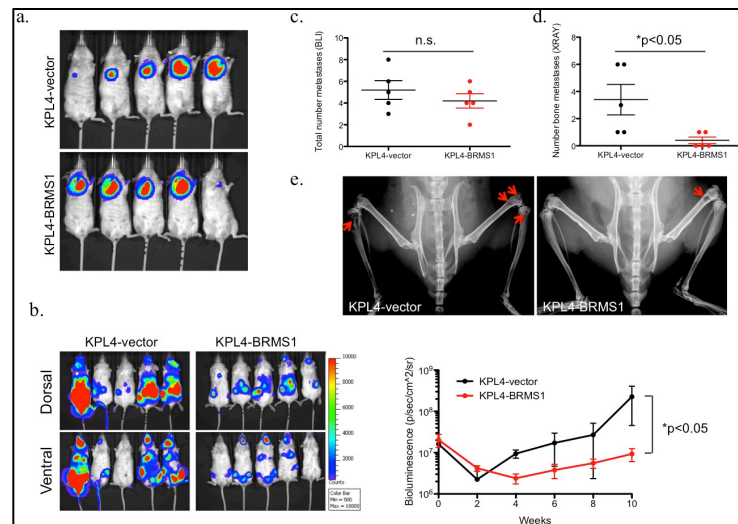


Figure 2. BRMS1 specifically inhibits bone metastasis of KPL4 breast cancer cells. a. 5×10^6 vector or BRMS1-expressing KPL4 cells were injected into a mammary fat pad of 3-4-week-old NOD/SCID mice. Tumor growth was monitored by weekly bio-luminescence imaging. b. 1×10^6 vector or BRMS1-expressing KPL4 cells were injected intravenously into 3-4-week-old NOD/SCID mice. Disease progression was monitored by weekly bioluminescence imaging and the results were quantified. Data shown are group mean values \pm SEM. Statistical analysis was performed utilizing Mann-Witney

test. c. Total number of metastatic lesions was counted per mouse based on bioluminescent images ($n=5$ per group). Data were analyzed by Student's t-Test. d. Total number of bone-specific metastatic lesions was counted per mouse based on whole body x-ray images ($n=5$ per group). Data were analyzed by Student's t-Test. e. Representative x-ray images show extensive osteolysis in bones of KPL4-vector-bearing mice, while few osteolytic lesions were detected in mice bearing KPL4-BRMS1 tumors.

Task 2.2.

Based on strong *in vivo* phenotype observed with KPL4-vector and KPL4-BRMS1 cells (Fig. 2), we then sought to determine a molecular mechanism responsible for suppression of bone metastasis. Since we also observed a concurrent suppression of adhesion of KPL4-expressing cells to several matrices (Fig. 1d) and protein kinases play an important role in regulating cell adhesion, we utilized a non-biased approach described in Task 2.2. We first performed a kinase array on regular cells grown in culture without going through the adhesion assay to determine which pathways were de-regulated in these cells simply through BRMS1 expression. Based on these results, we will then perform another array on post-adhesion samples.

By performing a kinase array that determines levels of phosphorylated kinases and few of their phosphorylated substrates, we were able to identify six differentially regulated targets whose expression is suppressed in BRMS1-expressing cells (Fig. 3a). None of the targets present on this array were expressed at a higher level in BRMS1-expressing cells.

Since SRC is one of the most well established kinases that regulates bone metastasis (4, 5) and adhesion, we attempted to validate array data by Western blot analysis using antibodies recognizing specific SRC phosphorylation and de-phosphorylation sites. However, we did not observe any significant differences in SRC phosphorylation levels (data not shown). Two other interesting targets identified by this array are ALK kinase and phospho-Stat3. ALK (anaplastic lymphoma kinase) is a novel oncogene that has high significance to inflammatory breast cancer, as it has been shown to be amplified or overexpressed in about 86% of patients with this disease (6). While little is known about the functions of this kinase, there is some evidence that it may regulate bone dissemination, and we will perform additional studies to determine if it might be involved in the observed phenotype. At the beginning, however, we decided to concentrate on the most deregulated target, which was phospho-Stat3. Stat3 is a transcription factor that is located downstream of many cytokine and growth factor receptors and regulates anti-apoptotic genes. It was also recently shown to regulate stem cell population in breast cancers (7). We first confirmed reduction of Stat3 phosphorylation in KPL4 cells by Western blot (Fig. 3b). To ensure that this signaling modulation was not cell line specific, we generated another HER2-overexpressing inflammatory breast cancer cell line, SUM-149, to express BRMS1, and confirmed that indeed, Stat3 phosphorylation level was also reduced (Fig. 3b). Since Stat3 is a transcription factor, we tested expression of several of its target genes: p21, IL-6, and IL-8. As expected, expression of p21 is typically suppressed by Stat3 and was elevated in BRMS1-expressing KPL4 and SUM-149 cells (Fig. 3b). Conversely, expression of IL-6 and IL-8, which are positively regulated by Stat3 signaling cascade, was reduced (Fig. 3c). Importantly, these two cytokines play an important role within the bone marrow microenvironment by promoting survival of endothelial cells and enhancing bone turnover, and may be partially responsible for suppression of growth of BRMS1-expressing cells in the bone. As described in the literature, Stat3 phosphorylation occurs in the cytoplasm and drives its translocation to the nucleus. However, despite a significant inhibition of Stat3 signaling, phosphorylated Stat3 translocated into nucleus of both vector and BRMS1-expressing cells at a similar rate (Fig. 3d), suggesting that there may be another method of regulating Stat3 transcriptional activity. Recent publications described mSin3a co-repressor complex in conjunction with HDAC1 to deacetylate Stat3, thereby disrupting its dimerization and inhibiting its transcriptional activity (8). Since BRMS1 is an integral member of mSin3a complex, it is possible that when it is expressed, the mSin3a complex is more stable or active, resulting in inhibition of Stat3 signaling.

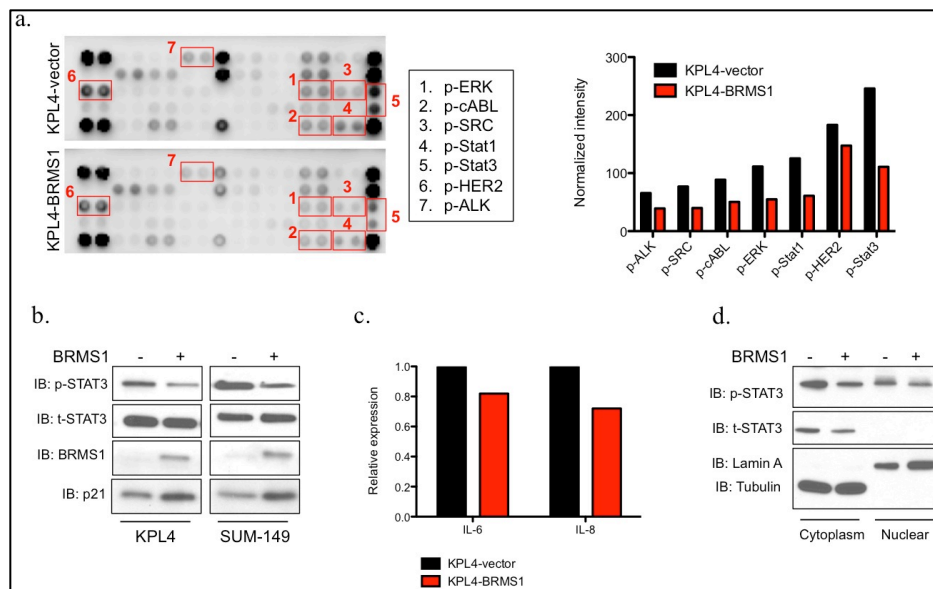


Figure 3. BRMS1 inhibits Stat3 phosphorylation and signaling. a. Vector and BRMS1-expressing KPL4 cells were lysed and lysates hybridized with membranes spotted with specific antibodies (antibody array). Membranes were developed by chemoluminescence. Results were quantified using ImageJ, and normalized intensity is shown in the bar graph. b. Vector or BRMS1-expressing KPL4 cells were lysed and lysates immunoblotted for proteins indicated. c. Total RNA was collected from vector and BRMS1-expressing KPL4 cells, and relative expression of IL-6 and IL-8 estimated using qPCR. Data are representative of two independent experiments. d. Lysate fractionation confirmed a reduction in cytoplasmic phospho-Stat3 levels, but similar phospho-Stat3 levels in the nucleus.

Task 2.4.

As we described in our Statement of Work, our ultimate goal is to identify potential therapeutic approaches capable of repressing adhesion and metastasis. Based on above results, our next objective was to investigate possible upstream regulators of Stat3 signaling, since there is currently no specific inhibitor of Stat3 function.

It is well known that Stat3 can be activated by a variety of growth factors and cytokines, so we tested ability of vector and BRMS1-expressing cells to respond to various stimulants. As shown in Figure 4a, vector cells responded to stimulation with basic FGF (bFGF) and heregulin (HRG), while BRMS1-expressing cells responded to HRG and EGF stimulation. Interestingly, even though BRMS1-expressing cells exhibited increased (as compared to untreated cells) Stat3 phosphorylation in response to these two stimuli, Stat3 phosphorylation still did not reach the level of unstimulated vector cells, again suggesting that there may be another mechanism that regulated Stat3 phosphorylation. Moreover, when we treated vector and BRMS1-expressing cells short-term with various inhibitors (SAHA: a broad HDAC inhibitor; Iressa: EGF receptor kinase inhibitor; Taxol: microtubule stabilizing agent, inflammatory breast cancer standard of care; MS-275: a new, selective HDAC1 and HDAC3 inhibitor), neither vector, nor BRMS1-expressing cells responded to EGF receptor signaling inhibition (Fig. 4b). Conversely, treatment with SAHA completely abolished Stat3 phosphorylation in BRMS1-expressing cells (Fig. 4b). On the other hand, short-term Taxol and MS-275 treatment resulted in enhanced Stat3 phosphorylation in vector cells, while Stat3 phosphorylation only increased upon treatment with MS-275 in BRMS1-expressing cells. Together, these data suggest that BRMS1 may regulate Stat3 signaling through HDAC1 activity, such as when HDAC1 activity is inhibited, at least short-term, inhibition of Stat3 is reversed. Our next step will be to test action of these inhibitors utilizing the adhesion assay of MDA-MB-231 and MDA-MB-435 vector and -BRMS1-expressing cells, followed by an *in vivo* metastasis assay.

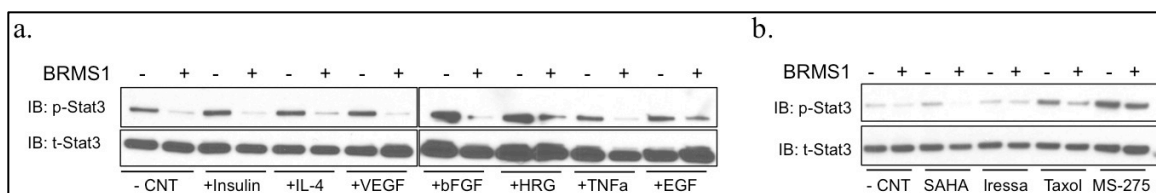


Figure 4. BRMS1 may regulate Stat3 signaling through HDAC1 activity. a. Vector and BRMS1-expressing KPL4 cells were serum-starved for 18 hours and then treated with stimulants indicated for 15 minutes. Cell lysates were collected and immunoblotted for phospho- and total levels of Stat3. b. Vector and BRMS1-expressing KPL4 cells were treated for 2 hours with drugs indicated in the presence of 10% sera. Cell lysates were collected and immunoblotted for phospho- and total levels of Stat3.

Task 2.3.

In addition to performing kinase antibody array, we also immunoprecipitated BRMS1 protein complexes for mass spectrometry analysis, reasoning that we could further investigate how BRMS1 may be regulating Stat3 activity, as well as to identify yet unknown cytoplasmic BRMS1-interacting partners (described in SOW Task 2.3). As shown in Figure 5, immunoprecipitation greatly enhanced BRMS1 signal intensity, and silver stain revealed several differentially expressed protein bands. We just received mass spectrometry data about 2 days ago and have not been able to verify any of the interactions yet. In total, there were 558 proteins identified from vector and BRMS1-expressing cells, while only 30 of them were preferentially identified in BRMS1-expressing cells. As expected, majority of these proteins were nuclear, a few were cytosolic, and several are known to be restricted to the plasma membrane. In addition, mass spectrometry determined that BRMS1 has a single phosphorylation site, indicating that there is another level of BRMS1 post-translational modification. Identification of this phosphorylation site is completely novel and we will validate these results in a near future.

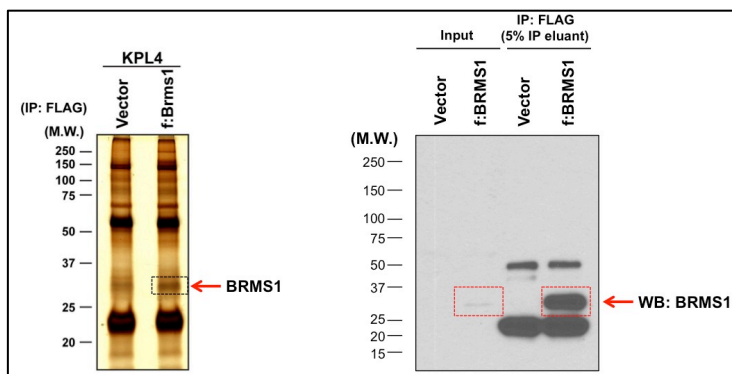


Figure 5. Preparation of samples for mass spectrometry. Following immunoprecipitation of FLAG tag, samples were resolved on SDS-PAGE and gels either silver stained (left panel) or immunoblotted with BRMS1 antibody (right panel).

Task 1.2.

To validate our results from BRMS1 overexpression system, during Year 1 we identified several breast cancer cell lines that endogenously express BRMS1 (described in SOW Task 1.2; Fig. 6a). Because BT474 cells grow extremely slowly in culture, we decided to use MCF7 cells for our knockdown studies. We screened 10 different shRNA clones targeting BRMS1 and determined that clones 1-4 exhibited an almost complete knockdown (Fig. 6b). We are now in the process of characterizing these cells and will utilize them to confirm BRMS1-mediated Stat3 regulation alterations.

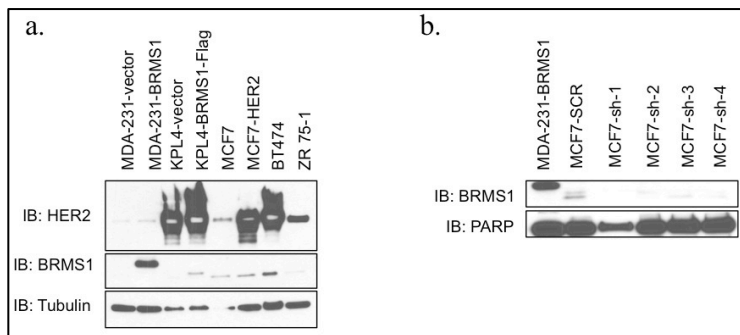


Figure 6. BRMS1 is endogenously expressed in several breast cancer cell lines. a. BRMS1 and HER2 expression were assessed by immunoblot in several breast cancer cell lines. b. BRMS1 was knocked down using shRNA-containing lentivirus.

3. Future work

3.1. We will determine the precise molecular mechanism by which BRMS1 inhibits Stat3 signaling.

3.2. We will determine if decrease in ALK phosphorylation detected in BRMS1-expressing cells is indicative of decreased ALK signaling cascade. We will test whether restoration of ALK will lead to restoration of bone metastatic phenotype in BRMS1-expressing cells.

3.3. We will verify novel protein-protein interactions of BRMS1 identified by mass spectrometry. Since several of the proteins are known to be localized in the cytosol, we will determine if alteration in their expression/cellular localization/activity is affected by BRMS1 and may be responsible for strong inhibition of cell adhesion and epithelial-to-mesenchymal transition observed in BRMS1-expressing cells (data shown in Year 1 progress report).

3.4. We will identify kinase responsible for phosphorylation of BRMS1 and determine biological functions of this post-translational modification.

4. Key Research Accomplishments

4.1. We fully characterized *in vitro* and *in vivo* behavior of vector and BRMS1-expressing KPL4 cells. We believe that this is the first time KPL4 cells were utilized to examine systemic dissemination of breast cancer. We determined that this cell line possesses capabilities to metastasize to ovaries, liver, lungs, kidneys, and bones following intravenous injection. We also observed skull-restricted bioluminescent signal from several mice, suggesting that these cells may be able to home to the brain, although we did not verify this observation histologically.

4.2. We identified that BRMS1 significantly inhibits Stat3 signaling, at least in the context of inflammatory breast cancer.

4.3. We identified several novel protein interactors of BRMS1, as well as a single BRMS1 phosphorylation site.

5. Reportable outcomes

5.1. Khotskaya YB, Sarvice MP, Shen J, Chang SS, Yu D, Steeg PS, and Hung MC. (2012) A novel model of breast cancer metastasis: killing two birds with one stone. AACR Annual Meeting, Chicago, IL

6. Conclusion

6.1. BRMS1 is a novel regulator of Stat3 signaling pathway.

6.2. BRMS1 interacts with several cytoplasmic proteins, suggesting that it may possess yet unknown functions.

6.3. BRMS1 can be phosphorylated, but the role of this phosphorylation is yet unknown.

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A novel model of breast cancer metastasis: killing two birds with one stone

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American women carry a lifetime risk of 1:8 of developing breast cancer, with metastatic disease remaining incurable. Between 70-80% of breast cancer patients diagnosed with metastases will exhibit lesions in their skeleton and in 25% of patients bone is the first site of metastasis. Breast cancer bone metastases are most frequently osteolytic. Thus, patients exhibiting bone lesions might suffer from nerve compression, bone fractures, hypercalcemia, pain, or paralysis. Existing treatment strategies for patients with bone metastases are palliative and designed to limit cancer-associated bone loss. It has been difficult to study breast cancer dissemination to bone due to lack of good animal models and little biopsy tissue availability. For human xenografts to establish bone lesions, human breast cancer cells are injected into the left ventricle of the heart, a procedure technically challenging and not 100% efficient. Resulting bone lesions are slow to develop and may be easy to miss on x-ray examination. Here, we show that a well-described parental MDA-MB-231 breast cancer cell line recapitulates progression of human disease when injected intravenously through the lateral tail vein into athymic nude mice. For reasons yet unclear, 30-50% of animals develop rapid and extremely lytic bone lesions in lumbar, sacral and caudal vertebrae and hind limbs. Similar to breast cancer patients, these animals exhibit pathologic fractures and paralysis, necessitating euthanasia within 4 weeks after cell inoculation. Interestingly, animals not affected by bone metastases go on to develop overt lung metastases within 10-12 weeks after cell inoculation. Bioluminescence imaging studies indicated presence of luciferase-tagged MDA-MB-231 cells in lungs of all mice at 2 and 24 hours post-cell inoculation. Within three weeks post-injection, large lesions were detected in the lumbar to caudal vertebrae regions and hind limbs of mice injected with luciferase-tagged (6/15 mice) or parental MDA-MB-231 cells (7/13 mice). Bone osteolysis was confirmed by x-ray, and paralysis occurred as early as 3 weeks after cell injection. Cytogenetic "finger printing" performed by the institutional core facility confirmed that cell lines used exhibit properties of MDA-MB-231 cells consistent with ATCC profile. Furthermore, molecular comparison of these cell lines with MDA-MB-231 cells obtained from other sources, including ATCC, revealed no differences. Unexpectedly, when examined in an orthotopic breast cancer model, MDA-MB-231 cells used in these studies produced rapidly growing tumors that spontaneously metastasized to lymph nodes and lungs, while MDA-MB-231 cells purchased from ATCC were completely non-tumorigenic. Studies are underway to determine a molecular mechanism responsible for this aggressive metastatic phenotype. This model may serve as a valuable tool for screening new therapeutics aimed to stop growth of metastases at multiple sites.

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